Strategies for purification of compounds from non-baseline separated peaks

Application

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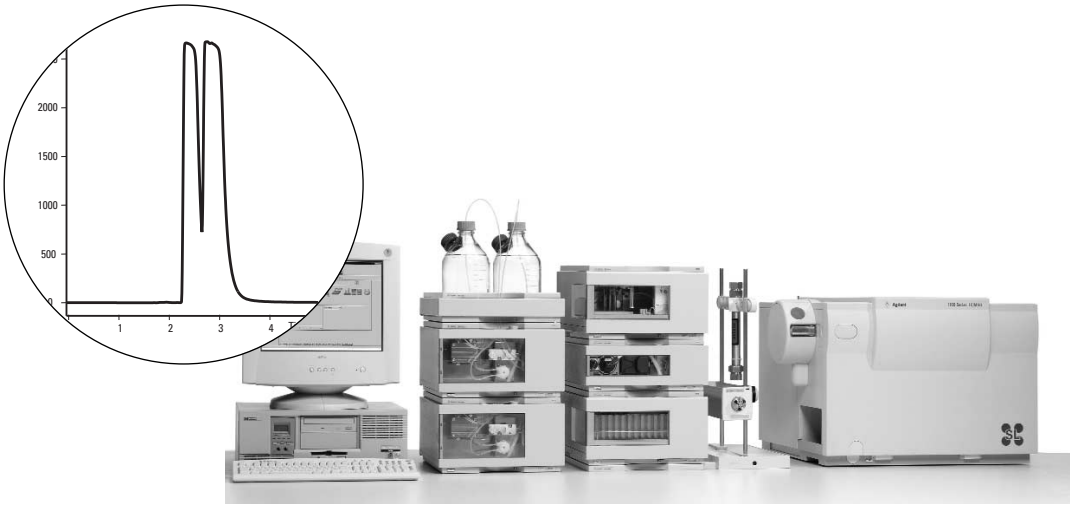

Abstract

In preparative HPLC chromatographic parameters such as resolution and peak shape may have to be sacrificed in order to achieve high purity and recovery of compounds in a reasonable throughput. Therefore, it may be necessary to purify compounds from non-baseline separated peaks in the chromatogram. In this Application Note we show four strategies how this task be can accomplished with the Agilent 1100 Series purification system[1,2] controlled by dedicated software.

Agilent Technologies

Introduction

For accuracy of quantitative results in analytical HPLC chromatographic parameters such as resolution and peak symmetry are essential. For preparative HPLC other, non-chromatographic parameters are important. These include recovery, purity and throughput. For example, to achieve high throughput, fast and steep gradients are used and the column is highly overloaded. This leads to non-baseline separated and unsymmetrical peaks. Despite the poor chromatography, good recovery and purity of the isolated compound is still desirable. To achieve this strategies have to be developed to purify compounds from non-baseline separated peaks. In this Application Note we describe four separation strategies:

1. Peak-based fraction collection with high threshold setting
2. Peak-based fraction collection using up- and down-slope
3. Peak-based fraction collection with time slicing of the peak
4. Mass-based fraction collection

Equipment

The system used included:

- Two Agilent 1100 Series preparative pumps
- Agilent 1100 Series preparative autosampler
- Agilent 1100 Series column organizer
- Agilent 1100 Series diode array detector
- Agilent 1100 Series fraction collector PS
- Agilent 1100 Series mass-selective detector (MSD)
- Agilent 1100 Series isocratic pump (as make-up pump)
- Agilent active splitter

The system was controlled using the Agilent ChemStation (rev. A.09.01) and the Purification/HighThruput software (rev. A.01.01).

Results and Discussion

1. Peak-based fraction collection with high threshold setting

The easiest way of doing peak-based fraction collection is the collection on threshold only. In the Agilent ChemStation and Purification/HighThruput software this can be done by deleting the parameters for up- and down-slope from the peak detector settings. This means a fraction is collected as soon as the detector signal exceeds a predefined value, and the collection ends when the signal falls below the specified threshold. This kind of peak-based fraction collection is sufficient for most purification applications. Non-baseline separated peaks can also be separated as shown in figure 1 but the threshold must be set to a rather high value. This leads to a cut-off of some of the compound at the beginning and the end of the peaks, which

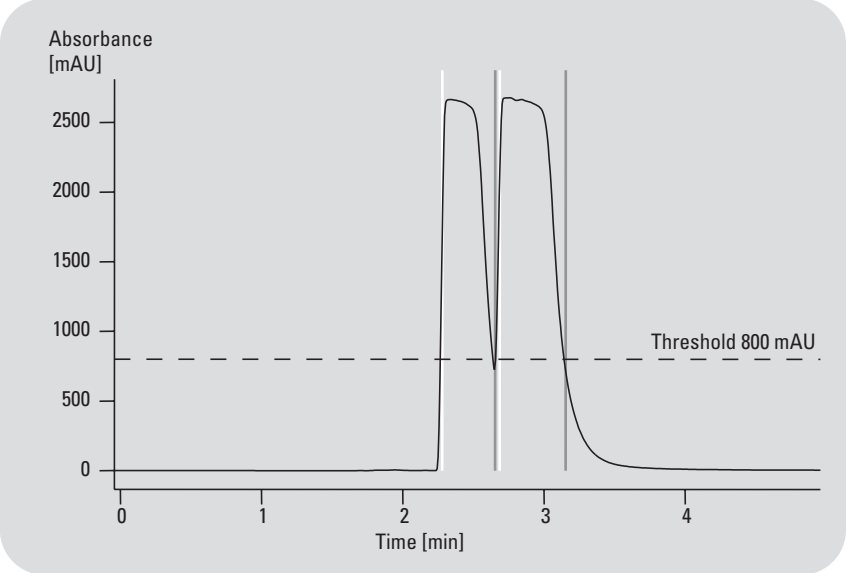

Figure 1
Peak-based fraction collection with high threshold setting

Columns	ZORBAX SB-C18 21.2 × 150 mm, 5 µm
Mobile phases:	A= water, B= acetonitrile
Isocratic:	40 % B
Stop time:	5 min
Flow rate:	20 mL/min
Injection:	750 µL
Column temp.:	ambient
UV detector:	DAD: 270/16nm (ref. 360/60 nm), preparative flow cell (3-mm pathlength)

decreases the recovery (see marked area in figure 2). The compound loss is not very significant for fast chromatography and rather steep peaks, however, for broader peaks it can be significant.

2. Peak-based fraction collection using up- and down-slope

To avoid compound loss at the beginning and the end of the peak the Agilent ChemStation and Purification/HighThruput software offers two additional parameters for peak-based fraction collection—up-slope and down-slope. When these parameters are set, a peak start is triggered if both parameters, up-slope and threshold, are exceeded. A peak stop is triggered if the signal meets one criteria, i.e. if it either falls below the threshold or below the specified down-slope value. As a result the threshold can be set to a rather low value, which allows to separate two non-baseline separated peaks into two fractions without losing compound at the peak start and end. This is shown in figure 3. To check the quality of the purification run, the two collected frac-

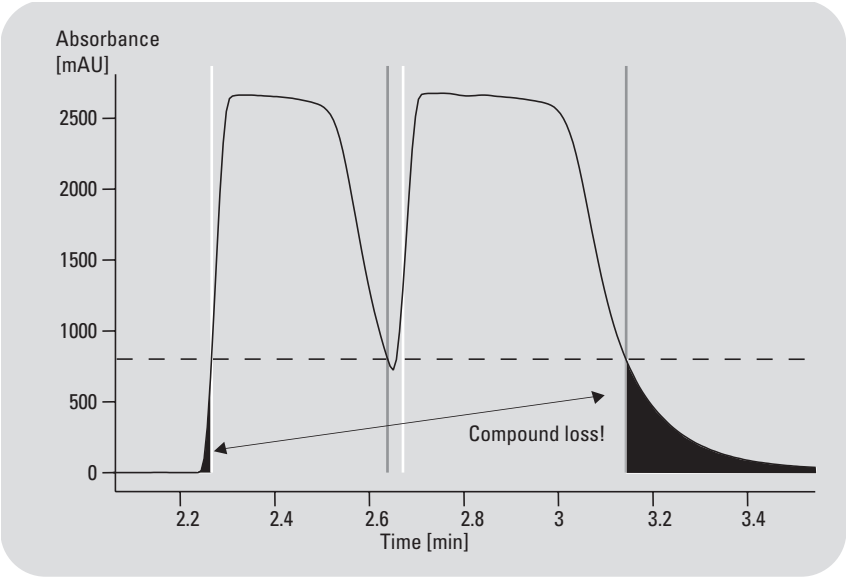

Figure 2
Compound loss due to high threshold setting

Columns	ZORBAX SB-C18 21.2 × 150 mm, 5 µm
Mobile phases:	A= water, B= acetonitrile
Isocratic:	40 % B
Stop time:	5 min
Flow rate:	20 mL/min
Injection:	750 µL
Column temp.:	ambient
UV detector:	DAD: 270/16nm (ref. 360/60 nm), preparative flow cell (3-mm pathlength)

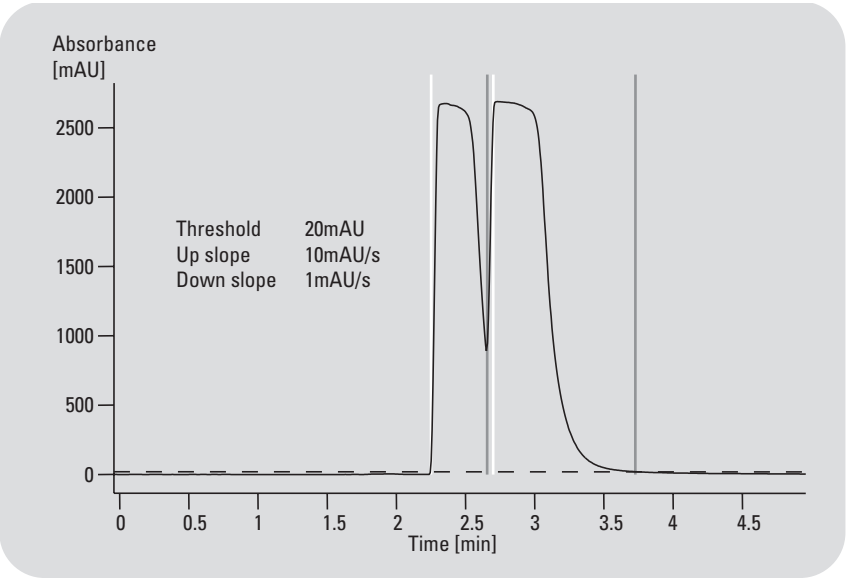

Figure 3
Peak-based fraction collection using up- and down-slope

tions were re-analyzed and the recovery and purity determined. Therefore, the analytical method was calibrated with standards of the two compounds. The results of the purification are summarized in table 1. It can be seen that, despite the poor chromatographic resolution the purification still leads to excellent results with pure compounds and recoveries over 90 %.

3. Peak-based fraction collection with time slicing of the peak

Peak-based fraction collection using up- and down-slope gives good recovery and purity as shown above. But if the overlapping peaks are very broad and therefore the overlapping area is large the two collected fractions are significantly impure. In this case a better strategy is to trigger the start of the fraction collection based on the signal but then cut the two peaks into several slices by time-based fraction collection. To accomplish this the *Max. fill volume per location* feature in the *Auxiliary* section of the *Setup fraction collector* window of the Agilent Chemstation software can be used (figure 4). With this feature the pre-configured fill volume of a fraction container can be changed within the method.

In the example shown in figure 5 deep-well plates were used as fraction containers and a fill volume of 2 mL was specified. Since the used flow rate was 20 mL/min a well fills up every six seconds. With the overall peak width of the two peaks of about 45 seconds the peaks are spread over eight wells. To avoid the loss of compounds when the fraction collection needle moves to the next collection location *Continuos flow* was configured as *Collection Mode*. In this mode, which is only configurable if well-plates are used as fraction containers, the diverter valve does not switch to the waste position when the collection needle moves to the next position.

The results of the fraction collection are shown in figure 5.

To decide which fractions can be combined and if any fractions that contain a mixture have to be re-purified all collected fractions were re-analyzed. Table 2 and figure 6 show the results and the reconstructed chromatograms of the compounds.

	Compound 1 [mg]	Compound 2 [mg]		
Fraction 1	10.97	0.00	Purity compound 1	100 %
Fraction 2	0.04	9.77	Purity compound 2	99.5 %
Recovery [mg]	11.01	9.77		
Recovery [%]	95.5	90.50		

Table 1
Results of peak-based fraction collection using up-and down-slopw

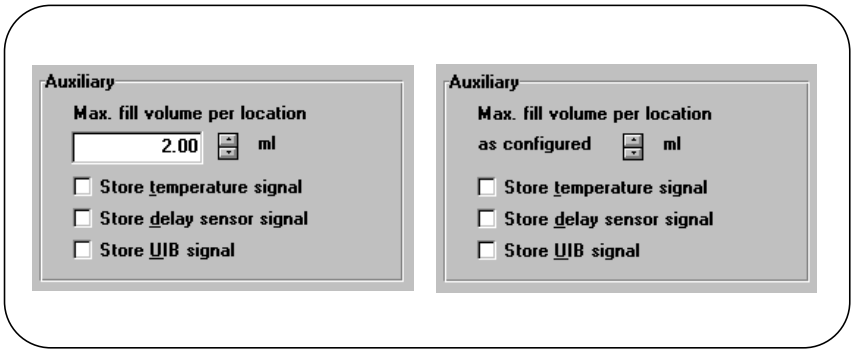

Figure 4
***Max. fill volume per location* settings**

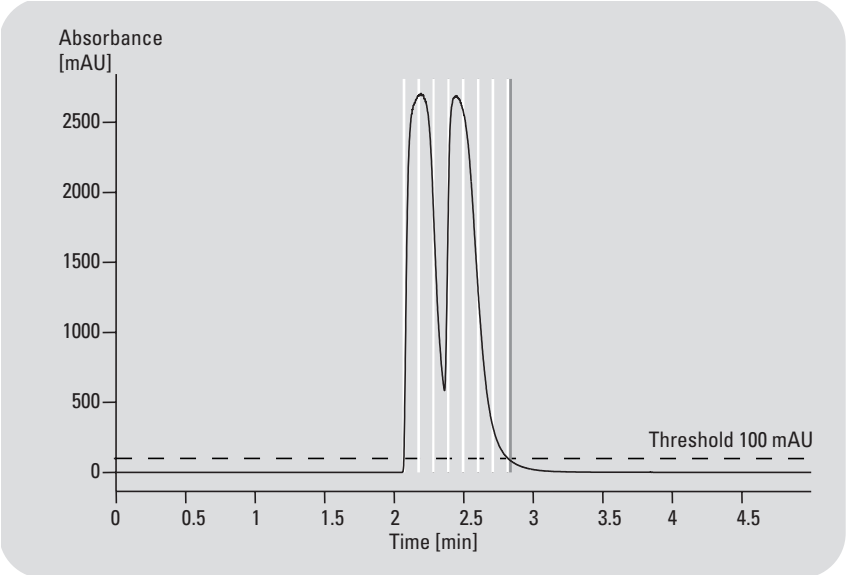

Figure 5
Peak-based fraction collection with time slicing of the peak

Columns	ZORBAX SB-C18 21.2 × 150 mm, 5 µm
Mobile phases:	A= water, B= acetonitrile
Isocratic:	40 % B
Stop time:	5 min
Flow rate:	20 mL/min
Injection:	750 µL
Column temp.:	ambient
UV detector:	DAD: 270/16nm (ref. 360/60 nm), preparative flow cell (3-mm pathlength)

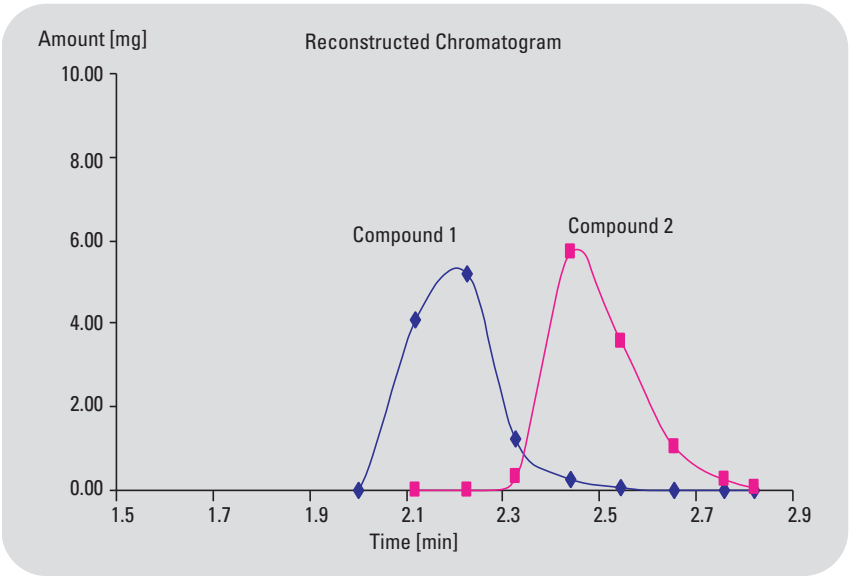

Figure 6
Re-constructed chromatogram

	Compound 1 [mg]	Compound 2 [mg]
Fraction 1	4.12	0.00
Fraction 2	5.20	0.00
Fraction 3	1.22	0.35
Fraction 4	0.25	5.73
Fraction 5	0.06	3.59
Fraction 6	0.02	1.03
Fraction 7	0.01	0.27
Fraction 8	0.00	0.03
Recovery [mg}	10.87	11.004
Recovery [%]	100.02	100.8

Table 2
Re-analysis of fractions

4. Mass-based fraction collection
Another possibility to purify compounds from non-baseline separated peaks is mass-based fraction collection. Since triggering is done on the target mass with the highest abundance a second fraction is started as soon as the target mass of the second peak becomes the dominant ion in the MS spectrum. The result of the fraction collection is shown in figure 7. Re-analysis of the collected fractions gave similar results for recovery and purity as for peak-based fraction collection using up- and down-slope as shown in table 3.

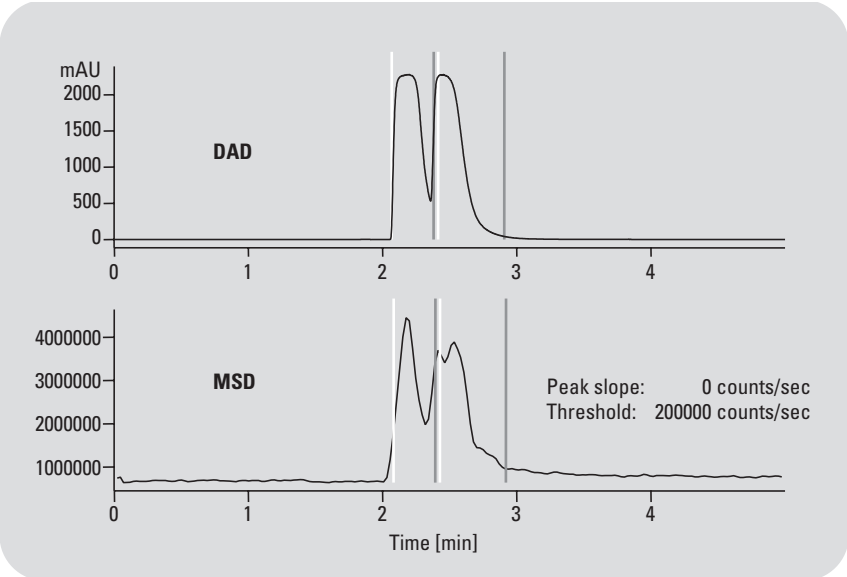

Figure 7
Mass-based fraction collection

Conclusion

In this Application Note we showed four strategies for the purification of compounds from non-baseline separated peaks. Peak-based fraction collection on threshold only (1) is the easiest way to separate the two compounds but the threshold has to be set high, which can lead to compound loss at the beginning and end of the peaks. This can be avoided by using the up-and down-slope features (2) of the ChemStation software. If the peaks are broad and are overlapping over a broad area the peak-based fraction collection with time slicing of the peaks (3) can be used. This requires some manual calculations regarding the proper fraction fill volume and will lead to a higher number of fractions. If a purification system equipped with an MSD is available mass-based fraction collection (4) can be performed, which also leads to only two fractions. Purity and recovery results are comparable with peak-based fraction collection using up-and down-slope.

	Compound 1 [mg]	Compound 2 [mg]		
Fraction 1	10.38	0.18	Purity compound 1	98.3 %
Fraction 2	0.25	9.97	Purity compound 2	97.5 %
Recovery [mg]	10.63	10.15		
Recovery [%]	97.80	92.90		

Table 3
Results of mass-based fraction collection

References

1.
"New perspectives in purification with HPLC and HPLC/MS", *Agilent Technologies Brochure*, **2001**, publication number 5988-3673EN.

2.
"Agilent 1100 Series Purification Platform — Customized system configurations", *Agilent Technologies Flyer*, **2001**, publication number 5988-3751EN.

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